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ISOLATION OF ANTIOXIDANT PRINCIPLE FROM *AZADIRACHTA* SEED KERNELS: DETERMINATION OF ITS ROLE ON PLANT LIPOXYGENASES

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An antioxidant principle was isolated from *Azadirachta indica* seed using high pressure liquid chromatography with a hydrophobic reverse-phase chromatography column. The eluted molecule had λ_{max} at 224 and 272 nm and was a potent inhibitor of plant lipoxygenases. In *in vivo* studies of horsegram during germination, low levels of lipoxygenase activity and lipid peroxides were found upon treatment with the *Azadirachta* extract. The antioxidant property of *Azadirachta indica* has not been previously reported.

Keywords: *Azadirachta indica*; Lipid peroxidation; Polyunsaturated fatty acids; Horsegram

Abbreviations: AA, arachidonic acid; ASAE, *Azadirachta* seed aqueous extract; ASE, *Azadirachta* seed extract; HPETE, hydroperoxy eicosatetraenoic acid; HPLC, high pressure liquid chromatography; LA, linoleic acid; LOX, lipoxygenase; LT, leukotriene; PUFAs, polyunsaturated fatty acids; LPO, lipid peroxides; Rt, retention time; NSAIDs, nonsteroidal antiinflammatory drugs.

INTRODUCTION

Azadirachta indica A. Juss (neem) is indigenous to the Indian subcontinent and its various parts are reputed as therapeutic agents.[1,2] Until today, the major part of research on neem is exclusively concerned with the use in agriculture, especially on insect control. In the medicinal use of traditional preparations, extracts from *A. indica* display a wide spectrum of uses. The

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empirical basics for the therapeutical use of the species have been laid down in classical texts of Ayurveda,[3,4] the ancient writing on skin diseases, inflammations and fevers.

Lipoxygenases (LOX: linoleate oxygen: oxido reductase; E.C.1.13.11.12) are nonheme-iron-containing dioxygenases that catalyze the hydroperoxidation of polyunsaturated fatty acids (PUFAs) containing a 1,4-pentadiene structure into conjugated hydroperoxy fatty acids. LOX activity has been found in a wide range of organisms, including plants, animals, fungi and cyanobacteria.[5–7] The physiological role of LOX in plant is poorly understood, but it has been suggested that LOX is involved in plant growth and development. Furthermore, LOX may also play a role in senescence, wounding, infection, pest resistance,[5–8] and in the formation of off-flavour/aroma in edible oils and vegetable foods.[9]

In animals, LOXs play several physiological roles; in particular, the product hydroperoxides are precursors of leukotrienes and lipoxins.[10,11] During the past decade it has become clear that higher levels of these compounds cause inflammatory or allergic diseases either acting alone or in combination.[12,13] Extensive research has been carried out to find effective inhibitors of LOX, but at present there are no practicable inhibitors for the treatment of inflammatory diseases except nonsteroidal antiinflammatory drugs (NSAIDs). Many of these drugs exhibit potent and specific inhibition of 5-LOX, but they are frequently attended by a variety of adverse effects either due to direct interference with other biological processes (e.g. methemoglobin formation in canine blood) or possibly as a result of by-production of reactive radical species by 5-LOX inhibition (for details, see review by Hutchinson *et al.*,[14]) for example, the induction of colitis by the effectiveness of 5-LOX inhibitors.[15,16] Currently antioxidants are widely used as LOX inhibitors, but they inhibit also the autooxidation of polyunsaturated fatty acids (PUFAs)[17,18] and only a limited number of safe antioxidants exist for human consumption. Therefore, a search for inhibitors from natural products having either oxygen scavenging or free radical trapping capabilities is required. In our laboratory, after finding inhibitory activity in *Azadirachta indica* extract for various legume and potato LOXs, we have been interested in isolating the active principle from *Azadirachta* to study its effect in limiting LOX activity and further peroxidation in plants.

MATERIALS AND METHODS

Arachidonic acid (AA) and linoleic acid (LA) were obtained from Sigma Chemical Co., St. Louis, MO, USA. The *Azadirachta* seeds were collected

locally and other seeds similar to ours were obtained from A.P. State Seed Corporation, Tirupati.

The legume seeds, sterilized with mercuric chloride, were washed, imbibed in water for 5 h and spread on petriplates for germination with a filter paper support. The horsegram seeds were allowed to grow in ASAE media (10% of 10 ml of ASAE provided per day) for treatment and deionized water was used for the control, and germinated under daylight for 12 h at $25 \pm 2°C$.

Enzyme Preparation

After 48 h a 10% homogenate of each germinating seedlings and potato tuber was prepared in 100 mM phosphate buffer containing 2 mM sodium metabisulphate, 2 mM ascorbic acid and 1 mM ethylene diamine tetraacetic acid (pH 6.4) with a Potter Elvejham homogenizer and was filtered through four layers of cheese cloth. The filtrate was centrifuged at $10,000 \times g$ for about 0.5 h. The supernatant was fractionated with ammonium sulphate to bring the concentration 30–60%. The active pellet obtained by centrifugation at $13,000 \times g$ was redissolved in 20 mM phosphate buffer, pH 6.4, and dialysed overnight against 150 vols of the same buffer with four changes. This partially purified fraction was used for the lipoxygenase assay. All the above steps were performed at 4°C. The protein content of the LOX was determined by the method of Lowry *et al.*[19]

Preparation of Substrate Solution

AA and LA stock solutions were prepared by the method of Reddanna *et al.*[20] The final concentration of AA and LA was 40 and 80 mM respectively, per millilitre absolute ethanol and aliquots stored under nitrogen gas at −20°C until used.

Enzyme Assays

Oxygen Consumption Method

The LOX activity in each species of seedlings was determined by the method of Reddanna *et al.*[20] using Gilson Oxygraph OXY-5 model. The typical assay contained air-saturated 150 mM phosphate buffer (1.9 ml) and the enzyme (10–100 µl) The reaction was initiated by the addition of substrate. The substrate concentration in the reaction mixture was 250 µM for LA or 133 µM for AA. After completion of the assay, a unit of LOX activity was expressed as µmoles of oxygen consumed per minute at 30°C.

Spectrophotometric Method

LOX activity was also measured by monitoring the absorbance at λ 234 nm due to the formation of hydroperoxy-conjugated diene in the presence of substrate and enzyme. The rate of the reaction was calculated by using a molar extinction coefficient of 27,500 for hydroperoxide. One unit of enzyme activity was defined as 1 μmol of hydroperoxide formed per minute at 30°C.

Preparation of *Azadirachta* Seed Extract

The 10% homogenate of *Azadirachta* seed kernel was prepared in distilled water or in pure methanol and stored at 4°C. After 24 h incubation the homogenate was centrifuged at $6{,}000 \times g$ for 20 min at 4°C. The supernatant, 10 ml day^{-1}, was used for inhibitory studies.

Isolation of the Active Principle of *Azadirachta* Seed Kernel on HPLC

Isolation of the active principle of *Azadirachta* seed kernel was carried out using Shimadzu HPLC ODS reverse-phase hydrophobic column. The HPLC column was equilibrated with acetonitrile : water (1 : 1 v/v) and the seed methanolic extract was injected into the HPLC. Isocratic elution was performed with the same solvent ($CH_3CN : H_2O$) at a flow rate of 0.5 ml/min. Each peak with absorbance at 215 nm eluted from the column was collected and examined for its inhibitory action on LOX and its UV absorption spectrum using a Shimadzu UV spectrophotometer.

Inhibition Studies

The *in vitro* effect of *Azadirachta* seed aqueous extract (ASAE) and HPLC fractions on LOX activity was measured after preincubation of an aliquot (300 μl) with the enzyme mixture for 2 min followed by substrate addition. Control experiments were carried out with addition of assay buffer in place of inhibitor.

Preparation and Isolation of Arachidonic Acid Products

To initiate the reaction, 135 μM of AA was added to the mixture containing 100 mg of partially purified LOX protein from germinating seedlings in assay buffer, in the presence and absence of ASAE. The mixture was mixed

vigorously and incubated for 2 min at 30°C. The reaction was terminated by acidifying the reaction mixture with 6 N hydrochloric acid. The products were extracted twice with hexane : diethylether (1 : 1 v/v). The organic extracts were pooled, dried over anhydrous sodium sulphate and evaporated to dryness by passing inert nitrogen gas. The UV absorption spectra (450–180 nm) for these extracts were obtained.

Lipid Peroxidation

Lipid peroxide levels of control and ASAE-treated seedlings of horsegram were determined by the method of Heath and Packer.[21] One gram of seedlings was homogenized in 5 ml of 20% trichloroacetic acid (TCA) and centrifuged at 10,000 × *g* for 5 min. To 1 ml of the supernatant was added 4 ml of 0.5% thiobarbituric acid in 20% TCA (TBA–TCA) and the mixture was boiled at 95°C for 30 min, then quickly cooled in ice bath, and centrifuged at 10,000 × *g* for 15 min. The absorbance of the supernatant was measured at 532 nm and the value for the nonspecific absorbance at 600 nm was subtracted. The concentration of MDA was calculated using its extinction coefficient of $155\,mm^{-1}\,cm^{-1}$ and the amount of LPO was expressed in μmoles of MDA per gram wet weight tissue.

RESULTS AND DISCUSSION

A comparison of the lipoxygenase (*in vitro*) activity of various seedlings untreated and treated with *Azadirachta* seed extract (Tables I and II) showed that the lipoxygenase activity of almost all seedlings including

TABLE I Lipoxygenase activities in control and ASAE-treated samples of various germinating seedlings

Source	*LOX activity* (units/mg)*		
	Control	*ASAE-treated*	*% Inhibition*
Greengram	0.5 ± 0.01**	0.1 ± 0.02***	80
Blackgram	3.4 ± 0.1	0.94 ± 0.07***	72
Cowpea	2.3 ± 0.1	0.90 ± 0.07***	61
Horsegram	0.65 ± 0.03	0.12 ± 0.03***	82
Field beans	1.8 ± 0.2	0.41 ± 0.02***	77
Pigeonpea	0.5 ± 0.05	0.06 ± 0.011***	88
Soybeans	1.1 ± 0.1	0.17 ± 0.02***	85
Potato	0.62 ± 0.04	0.08 ± 0.02***	87

*Units of activity of LOX are expressed in μmoles of O_2 consumed/min/mg protein.
** Values are mean ± SD of three replications.
*** $P < 0.001$ vs control.

TABLE II Inhibition of partially purified horsegram lipoxygenase activity by fractions isolated from *Azadirachta*

Fraction	*Activity* *	*% Inhibition*
Control LOX	4381 ± 191†	—
$CH_3CN : H_2O$ (1 : 1 v·v)‡	3126 ± 125***	29
Fraction 1 (Rt = 2.244)‡	3054 ± 218**	30
Fraction 2 (Rt = 2.590)	1017 ± 125***	77
Fraction 3 (Rt = 3.018)	3199 ± 125***	27
Fraction 4 (Rt = 7.645)	2690 ± 251***	39

*LOX activity is expressed in μmoles of HPETEs formed per minute at 30°C.
**$P < 0.01$; ***$P < 0.001$ vs control.
†Values are mean ± SD of three replications.
‡300 μl of each fraction used for inhibition of enzyme.

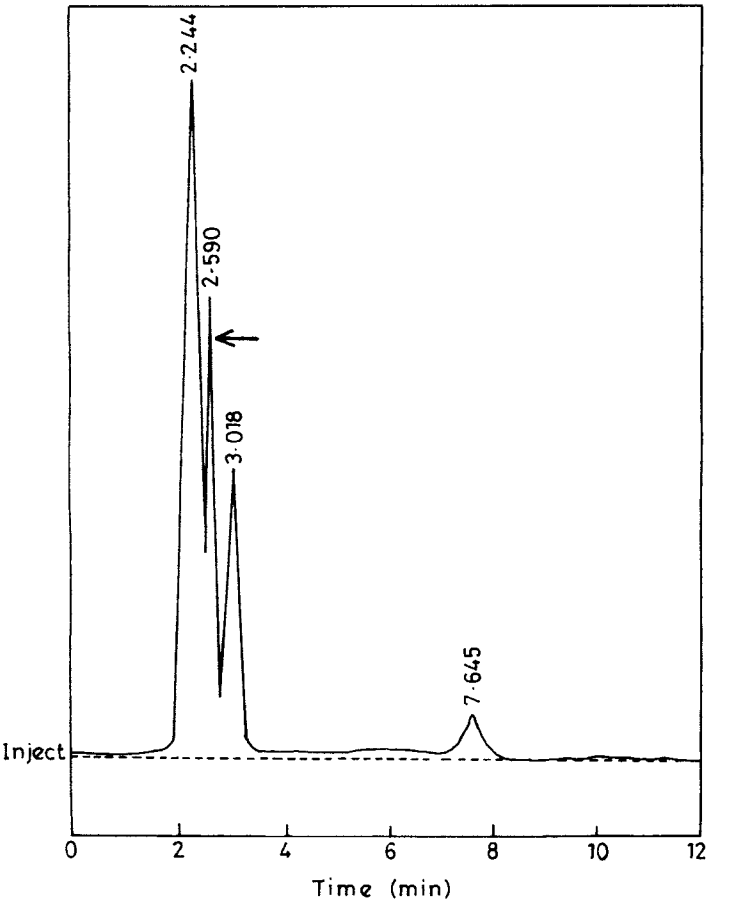

FIGURE 1A

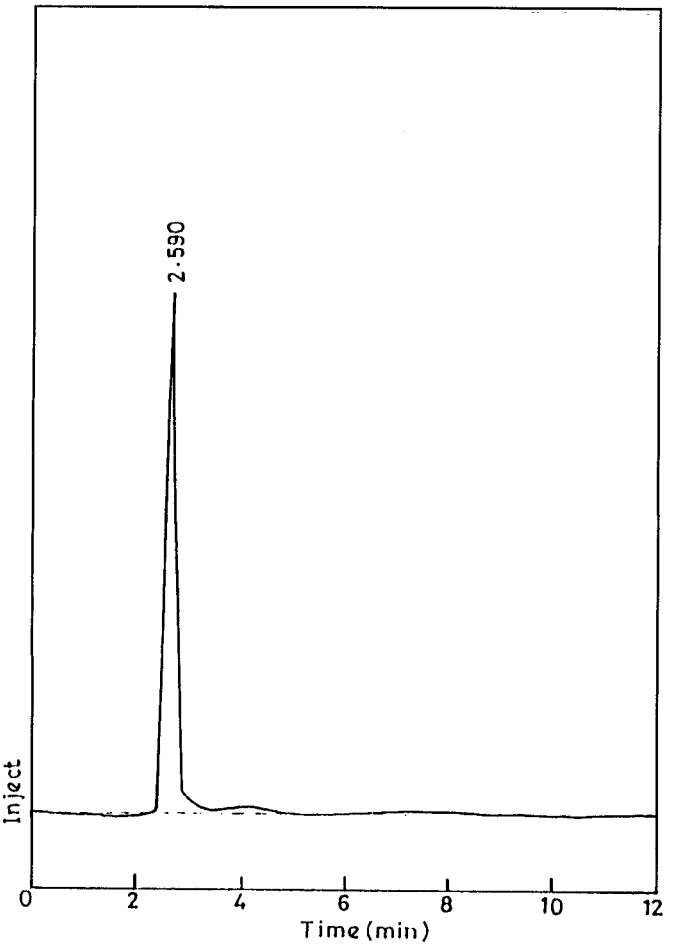

FIGURE 1B

FIGURE 1 (A) Reverse-phase HPLC chromatogram of *Azadirachta* seed kernel methanolic extract. (B) HPLC elution pattern of active fraction 2. (Isocratic run was performed using $CH_3CN : H_2O$ (1 : 1 v/v) at λ 215 nm at a flow rate of 0.5 ml/min.)

potato tuber was more than 80% inhibited by preincubation with ASAE. Among the HPLC fractions of ASE, only fraction 2 (Peak 2, Figure 1A and B) showed significant, more than 75% inhibition of the LOX activity; the other fractions showing inhibition similar to that of acetonitrile (Table II). Fraction 2 had absorption maxima at λ 224 and 272 nm (Figure 2).

Despite the fact that LOX from various sources differ in their mechanism of attack on unsaturated fatty acids, optimal reaction conditions and

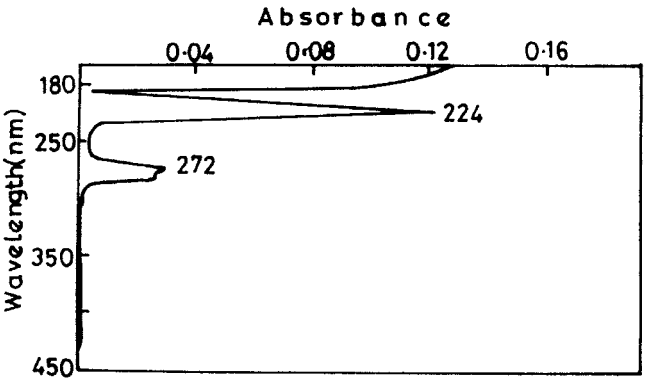

FIGURE 2 Spectroscopic scan of HPLC fraction 2, diluted (1 : 50) with $CH_3CN:H_2O$ (1 : 1 v v).

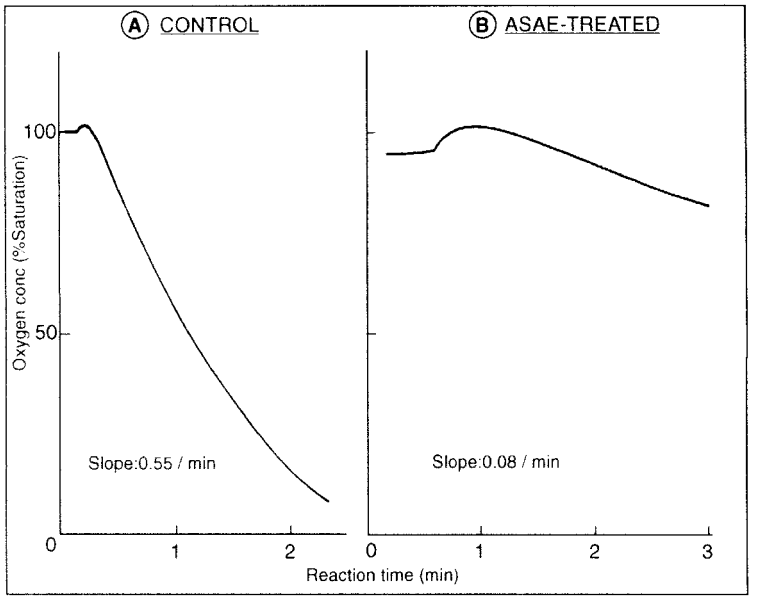

FIGURE 3 Typical oxygraphic assays of horsegram LOX (A) control (−ASAE) and (B) treated (reaction mixture was preincubated with ASAE).

product species, polarographic and spectrophotometric methods are commonly used to study LOX activity.[22] Accordingly we also used the oxygraph method to monitor the oxygenation activity in the sample with and without the inhibitor. Inhibition was again observed in the oxygen uptake rate with the inhibitor versus a control (Figure 3A and B). The inhibition of oxygen

uptake during the LOX reaction was dependent on the amount of ASAE added.

In spectroscopy, the products of the greengram LOX reaction showed two absorption maxima, one at λ 234 nm (HPETEs/HETEs) and the other at λ 276 nm (LTs), whereas the two peaks disappeared after ASAE treatment and the substrate absorption maxima appeared at λ 214 nm (Figure 4). Similarly soybean LOX, control and ASAE-treated, gave products with three peaks at λ 210, 235 and 280 nm and only one peak at λ 214 nm, respectively (Figure 4). From these results it is evident that the plant LOXs are unable to metabolize the substrates in the presence of ASAE. The results of LOX pre-incubation studies and oxygen consumption (Figure 3A and B) demonstrated that the *Azadirachta* seed extract (ASE) may block the function of LOX at both initial and subsequent stages.

Though a higher enzyme activity is found in blackgram, cowpea, field beans and soybeans (Table I), the horsegram has not been studied in detail. Therefore, we selected the horsegram and studied the levels of its lipid peroxides (LPO) and LOX and their inhibition during its seed germination.

Free radicals are produced continuously in cells as accidental by-products of metabolism or deliberately during some enzymatic reactions. Metal ions like iron can induce lipid peroxidation via a non radical mediated

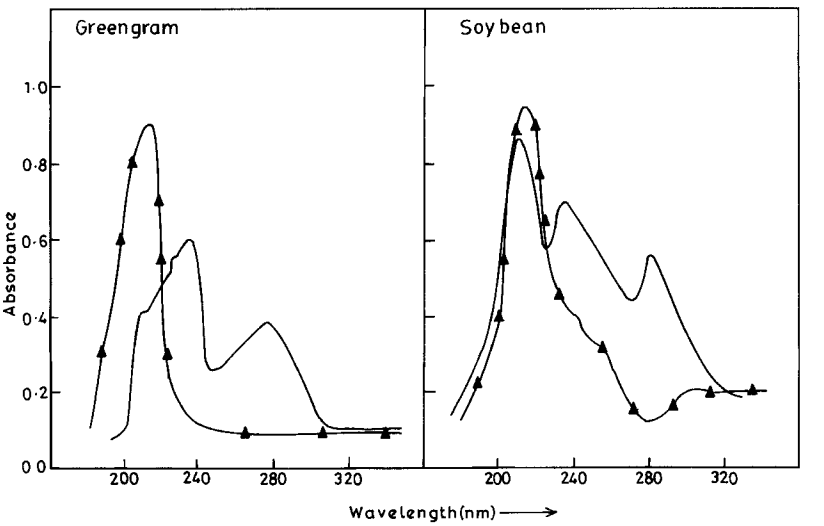

FIGURE 4 UV absorption spectra of greengram and soybean LOX products. The continuous line (—) indicates control (−ASAE), and discontinuous line (-▲-), treated (+ ASAE).

mechanism. The molar ratio of ferric ferrous state also plays a significant role in initiation and propagation of lipid peroxidation.[23] The LOXs are nonheme iron containing enzymes which play a significant role in metabolizing the membrane PUFAs to LPO through a free radical mediated reaction.[7,24] The LPO and LOX levels (*in vivo*) during germination of horsegram seedlings are given in Tables III and IV for both control and ASAE-treated. In the control, the LPO increased gradually as germination progressed until day 3 and thereafter remained more or less constant, whereas, in ASAE-treated seedlings, reduced lipid peroxidation and growth retardation (data not showed) were found. The LOX activity was elevated on day 3 in the control and in ASAE treatment and declined gradually thereafter. The growth retardation of seedlings in the presence of ASAE suggests that lipid peroxidation and its propagation by lipoxygenases are required for cell

TABLE III Lipid peroxide levels* in control (untreated) and ASAE-treated horsegram seedlings

Day of germination	*Control*	*ASAE-treated*†
1	7.0 ± 0.1**	6.0 ± 0.2 ***
2	8.0 ± 0.4	7.0 ± 0.1 ***
3	11.0 ± 0.3	10 ± 0.1 ***
4	10.0 ± 0.1	9.0 ± 0.2 ***
5	8.0 ± 0.2	8.0 ± 0.4
6	8.0 ± 0.4	7.0 ± 0.1 ****
7	8.0 ± 0.1	6.2 ± 0.4 ***

* Lipid peroxides are expressed in μmoles of MDA formed per gram wet tissue.
** Values are mean ± SD of three replications.
*** $P < 0.01$; **** $P < 0.02$ vs control.
†Horsegram was grown in *Azadirachta* media (10 ml of 10% *Azadirachta* seed kernel aqueous extract was provided per day).

TABLE IV Specific activity of LOX in control (untreated) and ASAE-treated horsegram seedlings

Day of germination	*LOX activity* (units/mg)*	
	Control	*ASAE-treated*†
1	3.2 ± 0.2**	2.4 ± 0.2 ***
2	5.8 ± 0.2	3.7 ± 0.4 ***
3	7.3 ± 0.2	5.8 ± 0.4 ***
4	6.5 ± 0.4	4.6 ± 0.4 ***
5	3.2 ± 0.1	1.7 ± 0.2 ****
6	2.4 ± 0.05	0.9 ± 0.03 ****
7	3.0 ± 0.4	1.2 ± 0.1 ***

* Units of activity of LOX are expressed in μmoles of O_2 consumed/min/mg protein.
** Values are mean ± SD of three replications.
*** $P < 0.01$; **** $P < 0.001$ vs control.
†Horsegram was grown in *Azadirachta* media (10 ml of 10% *Azadirachta* seed kernel aqueous extract was provided per day).

elongation and development,[6,7] and their prevention may cause damage to cell growth. It is believed that, during germination, oxygenases play an active role in PUFA oxidation, and the free radicals generated further propagate oxidation of PUFA which are then converted into sugars via gluconeogenesis to provide further metabolic energy in the cell.[25] Apparently these active LPO and LOX processes occurring in horsegram seedlings are inhibited by *Azadirachta* seed extract (Tables III and IV).

In summary, our studies on LOX and LPO in seedlings treated with *Azadirachta* seed extract showed that ASE acts as an inhibitor for both of them. The objective of the present study was to investigate the antioxidant property of neem since it possesses antiinflammatory and antiarthritic properties. The HPLC studies on ASE revealed that *Azaridachta* seed possesses an antioxidant to LOX and LPO. How this antioxidant can inhibit LOX and LPO and what it is will be studied and published elsewhere.

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References

[1] Dymock, W., Warden, C.J.H. and Hooper, D. (1890) *Pharmacographia India*, p. 89. The Institute of Health and Tibbi Research republished under the auspices of Hamdard National Foundation of Pakistan.
[2] Chopra, R.N., Nayar, S.L. and Chopra, I.C. (1956) *Glossary of Indian Medicinal Plants*, p. 31. CSIR; New Delhi.
[3] *Astangahrdaya Samhita* (1941) German translation by Hilgenberg, L. and Kirefl, W.E.J. Brill; Leiden, The Netherlands.
[4] *Caraka Samhita* (1949) Shree Gulabkunverba Ayurvedic Society, Jamnagar, India.
[5] Hildebrand, D.E. (1989) *Plant Physiol.*, **79**, 249–253.
[6] Siedow, J.N. (1991) *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **42**, 145–188.
[7] Gardener, H.W. (1991) *Biochim. Biophys. Acta*, **1084**, 221–239.
[8] Kato, T.K., Tanaka, K. and Shibata, D. (1992) *Plant Physiol.*, **98**, 324–330.
[9] William, D.C., Lim, H.M., Chem, A.O., Pangbornn, R.M. and Whitaker, J.R. (1986) *Food Technol.*, **40**, 130–140.
[10] Yamamoto, S. (1992) *Biochim. Biophys. Acta*, **1128**, 117–131.
[11] Samuelsson, B. (1983) *Science*, **220**, 568–575.
[12] Filep, J. (1989) Highlights of 2nd international conference on Leukotrienes and Prostaglandins in health and disease, October 9–14, 1988, Jerusalem. *Drugs Today*, **25**(2), 140–142.
[13] Forman, H.J. and Torres, M. (1991) In *Oxidative Damage Repair* (Int. Soc. Free Radical Res. Bienn. Meeting) 5th, Davis, K.J.A. (Ed.) pp. 636–641. Pergaman Press; Oxford.
[14] Hutchinson, A.W.F., Gresser, M. and Young, R.N. (1994) *Ann. Rev. Biochem.*, **63**, 383–417.
[15] Wallage, J.L., Mac Naughton, W.K., Morris, G.P. and Beck, P.L. (1989) *Gastroenterology*, **96**, 29–36.

[16] Vilaseca, J., Salas, A., Guarner, F., Rodriguez, R. and Malagelade, J.R. (1990) *Gastroenterology*, **98**, 269–277.
[17] Doba, T., Burton, G.W. and Ingold, K.V. (1985) *Biochim. Biophys. Acta*, **835**, 298–303.
[18] Liu, Z.L., Han, Z.X., Chen, P. and Liu, Y.C. (1990) *Chem. Phys. Lipids*, **56**, 73–80.
[19] Lowry, H.O., Rosenbrough, N.J., Farr, A.L. and Randal, R.J. (1951) *J. Biol. Chem.*, **193**, 265–275.
[20] Reddanna, P., Whelan, J., Maddipati, K.R. and Reddy, C.C. (1990) *Meth. Enzymol.*, **189**, 268–277.
[21] Axelrod, B., Cheesbrough, T.M. and Laakso, S. (1981) *Meth. Enzymol.*, **74**, 441–445.
[22] Heath, R.L. and Packer, L. (1968) *Arch. Biochem. Biophys.*, **125**, 189–198.
[23] Ryan, T.P. and Aust, S.D. (1992) *Crit. Rev. Toxicol.*, **22**, 119–141.
[24] Roy, P., Roy, S.P., Mitra, A. and Kulkarni, A.P. (1994) *Biochim. Biophys. Acta*, **1214**, 171–179.
[25] Beevers, H. (1979) *Ann. Rev. Plant Physiol.*, **30**, 159–193.